IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Andreas EHLICH *et al.*	Confirmation No.: 5698
Appl. No. 10/594,177	Art Unit: 1633
Filed: August 13, 2007	Examiner: Kelaginamane HIRYANNA
For: **SECRETED PROTEINS AS MARKERS FOR CELL DIFFERENTIATION**	Atty. Docket No.: 0066-0007-US1

Declaration of Dr. Andreas Ehlich Under 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

1. I, Dr. Andreas Ehlich, hereby declare and state as follows:

2. I am one of the named inventors of U.S. Application No. 10/594,177 (hereinafter "the '177 application"), filed September 25, 2006, entitled "Secreted Proteins as Markers for Cell Differentiation." I am also an employee of the assignee of the '177 application, Axiogenesis AG.

3. I hold the degree of Doctor of Philosophy. A recent copy of my Curriculum Vitae, accurately listing my scientific credentials and work experience is attached herewith as Exhibit A.

4. I have read and understand claims 1, 4, 6-9, 11-12 and 55, currently pending in the '177 application. I have also read and understand the Office Action, dated October 11, 2011.

5. The Office Action has rejected claims 1, 4, 6-9, 11-12 and 55, of the '177 application as obvious over Benkel *et al*, WO 98/49320 (hereinafter "Benkel"), in view of

Goldspink *et al.*, US 2003/0008836 (hereinafter "Goldspink") and Bronstein *et al.*, Biotechniques 17:172-177 (1994) (hereinafter "Bronstein").

6. The Office Action suggests that Benkel discloses the advantages of using a reporter gene system for studying the regulation of gene expression that is of fundamental importance to cell division and cell differentiation; and reporter genes whose expression product is secretable and used to monitor cell division and cell differentiation. Benkel allegedly discloses that there are several secretable reporter systems that include secreted alkaline phosphatase (SEAP), alpha-amylase, hGH; and teaches the use of a reporter construct including coding a secretable alpha-amylase with a signal peptide coding region and tissue of cell type specific promoters. The Office suggests that Benkel further discloses that the use of a secretable alpha-amylase gene is superior to other secretable reporters as the range of available variants of alpha-amylase allows the assays to be performed in virtually any host without interference.

7. The Office Action further suggests that Goldspink discloses a method of detecting myoblast differentiation by transfecting recombinant nucleic acid molecules encoding a human alpha-galactosidase reporter gene under the control of a promoter comprising an MCL1/3 enhancer into undifferentiated myoblasts wherein the reporter gene was expressed and secreted from differentiated muscle cell in an in vitro culture.

8. The Office Action further suggests that Bornstein teaches improvements in the detection of sensitivity of SEAP reporter using chemiluminescent assays of the secreted reporter from cells in culture or tissue.

9. The Office Action concludes that it would have been obvious to one of ordinary skill in the art to incorporate the SEAP reporter gene of Benkel for the lacZ gene in

the reporter construct of Goldspink and follow the differentiation of stem cells to specific tissue types or cell types using the very sensitive SEAP assays taught by Bornstein. The Office Action further concludes that one of ordinary skill in the art would have been motivated to make and use an assayable secreted reporter that will not be captured by tissues or the cells for monitoring a gene regulation during differentiation of a cell into tissue cells types as it is less invasive and avoids lysis of the cells. Finally, the Office Action concludes that one of ordinary skill in the art would have had a reasonable expectation of success in using a recombinant progenitor or stem cell having a reporter gene construct that codes for a secretable reporter protein for evaluating and identifying the differentiated cells as the art teaches that it is routine to use a recombinant secretable reporter for marking differentiation. I respectfully disagree with the Examiner's characterization and conclusions.

10. Although Benkel describes a secreted reporter gene system based on one or more alpha-amylases and describes that the alpha-amylase reporter gene encodes a biological activity that can be easily measured by a variety of liquid or semi-solid phase assay systems, Benkel does not describe a method for determining the effect of a test compound on the ability of pluripotent embryonic stem cells to undergo differentiation comprising measuring the activity or amount of a secreted reporter gene product; correlating the amount of reporter gene product with the proportion of differentiated cells; and comparing the amount of differentiated cells in the presence of the test compound to the amount of differentiated cells in a control sample.

11. Benkel distinguishes between *transformed* mammalian cells versus *untransformed* mammalian cells through the electrophoretic detection of secreted alpha-

amylases. In fact, each of the cell lines transformed in the Benkel application have already been differentiated.

12. The present invention, however, is directed to determining the effect of a test compound on the *differentiation* of pluripotent embryonic stem cells by measuring the amount or activity of a secreted reporter gene product.

13. Prior to the filing date of the present invention, the effect of a test compound on the differentiation of embryonic stem cells was primarily determined using the embryonic stem cell test (EST).

14. The embryonic stem cell test (EST) was the only *in vitro* system for embryotoxicity prediction that was successfully validated by a regulatory agency, namely the European Center for the Validation of Alternative Methods (ECVAM; Bremer and Hartung, 2004).

15. The EST assay relies on *in vitro* differentiation of mouse embryonic stem cells (ESCs). ESCs are first aggregated into embryoid bodies (EBs). After 8 to 14 days in culture, rhythmically contracting areas develop inside the EBs due to the development of cardiomyocytes during the differentiation process. The EST assay uses microscopic evaluation of the EBs at the end of the differentiation process to determine the proportion of EBs that show contracting areas. The embryotoxic potential of a test compound is determined based on its ability to alter that proportion relative to controls, (Scholz et al., 1999).

16. The EST assay validated by ECVAM has significant shortcomings (Bremer et al., 2002, Pellizzer et al., 2004, Marx-Stoelting et al., 2009). The EST assay allows throughput of only few substances. This is primarily due to the large amount of manual

work associated with the tedious visual inspection of EBs at the end of the differentiation period. Inspection of at least 24 EBs is required per data point and contractile areas are often difficult to detect, especially if they are small. Moreover, contractions in EBs tend to cease at temperatures below 30°C.

17. Our laboratory previously developed an assay that relies on the expression of a green fluorescent protein (GFP) reporter gene. GFP is specifically expressed by cardiomyocytes developed during in vitro differentiation of ESCs. Upon completed ESC differentiation, a cell lysate is prepared and the GFP fluorescence is determined by photometry, giving a measure of the amount of cardiomyocytes present in the EBs. Thus, visual inspection of individual EBs for contracting areas is avoided. This assay was used to test the embryotoxic potential of dental restorative materials using the GFP assay (Schwengberg et al., 2005).

18. The use of the GFP assay resulted in at least a tenfold increase in throughput when compared to the EST assay. However, the dynamic range of the GFP assay was quite limited and the assay required the determination of about 60 EBs per data point and counting of surviving EBs prior to measurement, due to variations in the number of EBs per sample. Moreover, use of the GFP reporter resulted in a low signal-to-background ratio.

19. The other Inventors of the '177 application and I developed a new and different assay for determining embryotoxicity of differentiation of ESCs. The claimed method is exemplified by an assay which involves the in vitro differentiation of ESCs carrying the SEAP reporter gene under the control of the alpha-myosin heavy chain promoter, resulting in cardiomyocyte-specific SEAP expression. First, we determined that cardiomyocytes were able to develop from single EBs cultured in a 96-well-plate. (Figure

1). Moreover, we determined that the amount of SEAP secreted was sufficiently high to allow analysis of such single EBs differentiated in individual wells of a 96-well-plate.

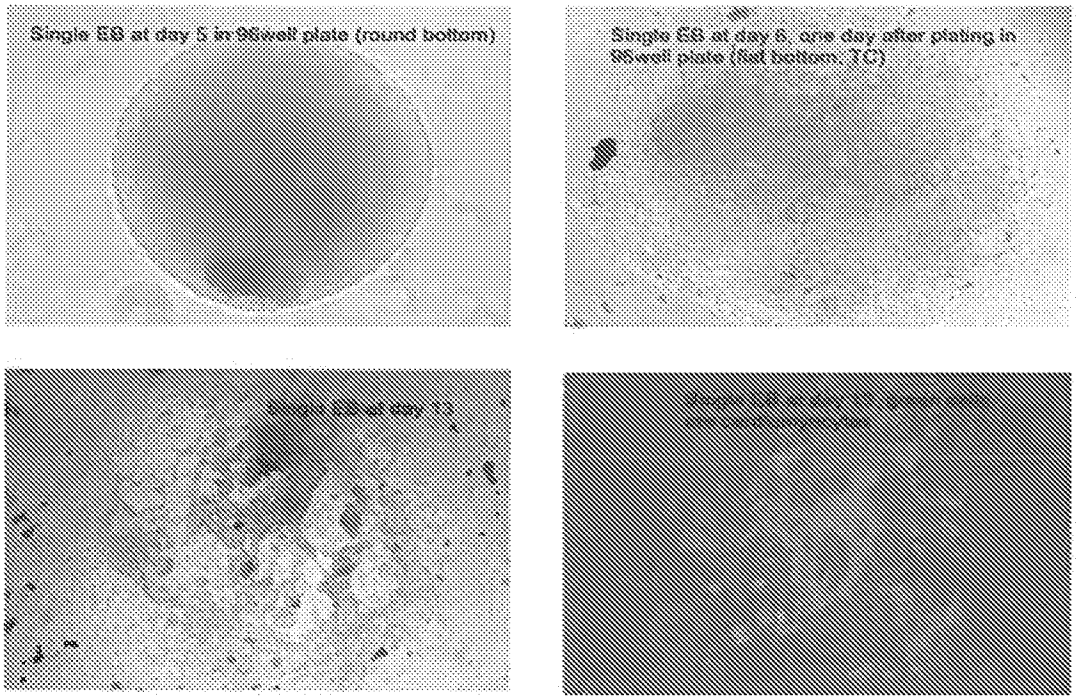

Figure 1. Cardiomyocyte development in EBs cultured individually in 96-well-plates. Single EBs were deposited into wells and observed at times indicated. In this case, the ESCs contained the cardiac-specifically expressed GFP reporter in order to visualize cardiomyocyte development.

20. The results that we obtained using SEAP to determine the effect of a test compound on the *differentiation* of pluripotent embryonic stem cells were surprising and unexpected. It was particularly surprising and unexpected that the signal-to-background ratio of the assay carried out as claimed was increased by more than a hundred-fold over the previous GFP assay. Furthermore, it was also surprising and unexpected that the amount of SEAP secreted was sufficiently high to allow analysis of single EBs.

21. Table 1A shows the enzymatic activities detected in the supernatants of individual EBs. The left column (marked "yes") contains values obtained from normally developed, single EBs. The second column (marked "no") indicates SEAP activities detected in supernatants of single EBs whose cardiomyocyte differentiation was completely inhibited by the presence of 10^{-5} M methylmercury and represents the background of the assay. As shown in Table 1A, the signal-to-background ratio was greater than 500.

22. Table 1B shows the corresponding experiment using the GFP reporter system described above. In the GFP assay, at the beginning of differentiation each sample contained about 60 EBs, because GFP fluorescence could not be determined from smaller samples. It was determined that the fluorescence signal obtained from EBs with normally developed cardiomyocytes was only about twofold stronger than the signal obtained from EBs without cardiomyocytes. Although efforts have been taken to improve the GFP signal-to-background ratio, that ratio still remains lower than ten.

A

	SEAP activity [μU per EB]	
	Cardiomyocyte differentiation	
Sample ID	yes	no
1	2987	4.7
2	2175	4.7
3	3340	4.2
4	1714	3.8
5	2571	4.2
6	2604	5.5
7	1406	4.2
Mean	2399	4.5
Ratio of Means		533

B

	GFP Fluorescence [per EB normalized, arbitrary units]	
	Cardiomyocyte differentiation	
Sample ID	yes	no
1	68.4	38.7
2	57.9	12.7
3	53.7	18.6
4	81.7	41
5	82	37.4
6	68.8	21.2
7	61.2	35.2
Mean	67.7	29.2
Ratio of Means		2.3

Table 1. Comparison of SEAP and GFP reporters to detect cardiomyocytes derived from ESCs by in vitro generation. SEAP and GFP reporter genes, respectively, for cardiomyocyte-specific reporter expression were introduced into undifferentiated ESCs. ESCs where aggregated into EBs and deposited in multiwell plates. In the case of cells with SEAP reporter, single EBs were cultured in wells of a 96-well-plate. For cells with the GFP reporter, 60 EBs were cultured in each well of a 6-well-plate. In both cases, half of the wells received 10^{-5} M methylmercury chloride which blocks development of cardiomyocytes completely. EBs were allowed to differentiate for 10 days. In the case of SEAP containing cells, supernatants were then collected and analyzed for SEAP activity. For GFP containing cells, numbers of surviving EBs was determined, EBs were lysed, and lysates were analyzed for GFP fluorescence. Values for cells differentiating in the absence of methylmercury chloride (yielding normal cardiomyocyte development) are given in columns on the left (Cardiomyocyte differentiation: yes); values for cells differentiating in the presence of methylmercury chloride (blocking cardiomyocyte differentiation) are given in right-hand columns (Cardiomyocyte differentiation: no).

23. We also determined if the SEAP reporter system was able to discriminate embryotoxic from non-embryotoxic compounds. Figure 2 shows the results obtained with two non-embryotoxic substances (penicillin and dimethyl phthalate) and four embryotoxic substances (methotrexate, hydroxyurea, methylmercury, and thalidomide). While penicillin and dimethyl phthalate had no influence on SEAP activity in the assay, the presence of each of the embryotoxic substances tested led to a concentration-dependent decrease in SEAP activity. These data indicate that the SEAP reporter allows detection of developmental toxicities of substances in the cellular differentiation assay.

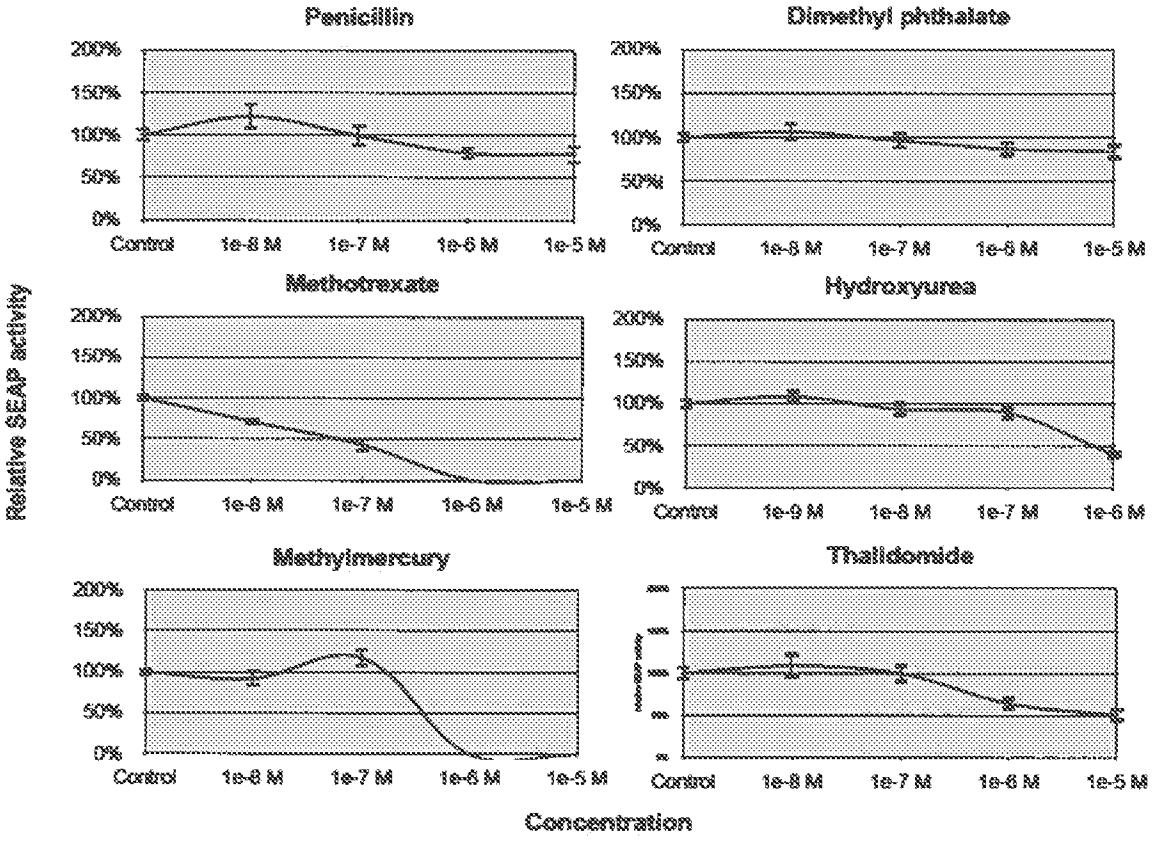

Figure 2. Effect of non-embryotoxic and embryotoxic substances on in vitro differentiating ESCs carrying a cardiomyocyte-specifically expressed SEAP reporter. Reporter transgenic ESCs were aggregated into EBs and single EBs were seeded onto a 96-well-plate and allowed to differentiate in the presence of substances indicated. Subsequently, supernatants were analyzed for SEAP activities. Values were normalized to samples receiving no substance, indicated as "controls".

24. In summary, the read-out using a GFP reporter provided advantages over the counting of contractile areas in the EST assay, because it increases the throughput by about tenfold. However, as noted above, the GFP-based assay still required the evaluation of about 60 EBs per data point and counting of surviving EBs before measurement, due to variation of EBs the samples and the provided low signal-to-background ratio.

25. These problems with the EST and GFP assays were surprisingly and unexpectedly overcome by employing a differentiation assay based on a secreted reporter gene product (e.g., SEAP). According to this assay, individual EBs can be analyzed and,

most importantly, the signal-to-background is increased by more than hundredfold, resulting in a surprising and qualitative increase in the assay sensitivity. Moreover, all steps of the differentiation assay employing SEAP can be automatized using robotic systems that are well available. Thus, individual EBs can be deposited into wells of a 96-well-plate using large-particle-flow-cytometry technology and the assay can be fully automatable.

26. The fact that the methods claimed in the '177 application exhibit a significant increase in sensitivity over the prior assays for determining the toxicity of a compound on differentiation of ESCs, I believe demonstrates a surprising and unexpectedly improvement that could not have been expected at the time of filing the '177 application.

27. I further state that all statements made on my own knowledge are true and that all statements made on information and belief are believed to be true and further that willful false statements and the like are punishable by fine or imprisonment or both under Section 1001 of Title 18 of the U.S. Code and may jeopardize the validity of the application or any patent issuing thereon.

March 7, 2012
Date

Dr. Andreas Ehlich

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CURRICULUM VITAE

Andreas Ehlich Ph.D.

Nattermannallee 1, D-50829 Cologne, Germany
T +49-221-998818-34 F +49-221-998818-10
andreas.ehlich@axiogenesis.com

Professional Experience

Senior Scientist, Axiogenesis AG, Cologne
New Development Division — 2001 to present

Group Leader, University of Cologne
Institute of Genetics — 1996 to 2001

Post-doctoral Fellow, University of Cologne
Laboratory of Professor Klaus Rajewsky, MD — 1995 to 1996

Education

Ph.D. Immunology and Genetics
University of Cologne — 1995

Diplom Biology (Corresponding to M.S. Biology)
University of Cologne — 1990

Vordiplom Biology (Corresponding to B.S. Biology)
Rheinisch-Westfälische Technische Hochschule Aachen, Germany — 1986

Selected Publications

Abassi, Y.A., Xi, B., Li, N., Ouyang, W., Seiler, A., Watzele, M., Kettenhofen, R., Bohlen, H., Ehlich, A., Kolossov, E., et al. (2012). Dynamic monitoring of beating periodicity of stem cell-derived cardiomyocytes as a predictive tool for preclinical safety assessment. Br. J. Pharmacol. *165*, 1424–1441.

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Research and Development Activities and Expertise

I lead R&D at Axiogenesis AG concerning in vitro generation of specific cells and tissues from mouse embryonic stem cells and am intimately familiar with the generation of cardiomyocytes, melanocytes, smooth muscle cells, and endothelial cells. My team is working on modification of such specified cells with reporter genes to make them suitable for screening purposes in the drug development process. Moreover, we employ these cells for modelling of human diseases.

March 7, 2012